Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Mendell JR, Campbell K, Rodino-Klapac L, et al. Dystrophin immunity in Duchenne's muscular dystrophy. N Engl J Med 2010;363:1429-37.

Supplementary Detailed Methods.

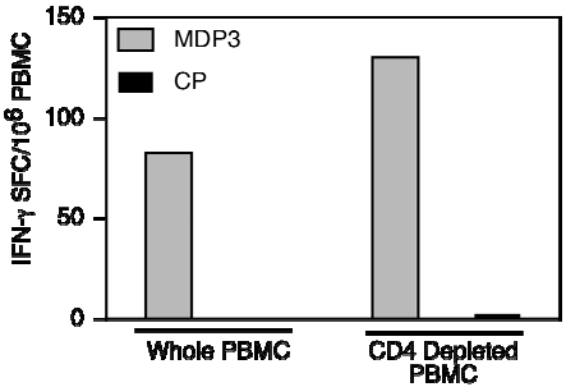
Vector. The genome of the rAAV vector used in this study encoded the amino-terminal actin binding domain (ABD), 5 rod repeat domains (R1, R2, R22, R23, and R24), 3 hinge domains (H1, H3 and H4), and the cysteine-rich (CR) domain of the human dystrophin gene. The human cytomegalovirus immediate early promoter regulated transgene expression. Vector genomes were packaged in AAV2.5, a serotype 2 capsid variant that contains five AAV1 amino acids (one insertion and four substitutions) in the AAV2 VP1 background. AAV2.5 offers improved muscle transduction properties of AAV1 with minimal recognition by serum neutralizing antibodies.

ELISpot assay. Peripheral blood T cell responses to minidystrophin were quantified by IFN-γ ELISpot assay. Briefly PBMC isolated on Ficoll hypaque gradients were cultured with synthetic peptides (20 amino acids in length, overlapping by 10 residues) that spanned the minidystrophin protein. Peptides were organized into 3 pools designated MDP-1, MDP-2, and MDP-3. MDP-1 represented the mini-dystrophin actin binding domain, H1 and R1 (exons E1 – E11/E12) and MDP-2 represented R2, R22, R23 and H3 (exons E12, E50-51, and E56-59). MDP-3 represented R24, H4 and the cysteine repeat region (exons E59-70). To identify individual peptides within a pool that elicited IFN-γ activity, so that each peptide was present in two of the intersecting mapping subpools. After incubation at 37^{o}C for 36 hr IFN-γ spot forming cells (SFC) were counted. Fewer than 10 SFC/well were observed with peptides from a control pool (CP) (enhanced green fluorescent protein). Responses were considered positive when SFC exceeded 50 per 10^6 PBMC in duplicate wells. In some cases mini-dystrophin-specific T cell lines derived from the blood of subjects 002 and 005 were used to fine map epitopes and determine HLA class I or II restriction.

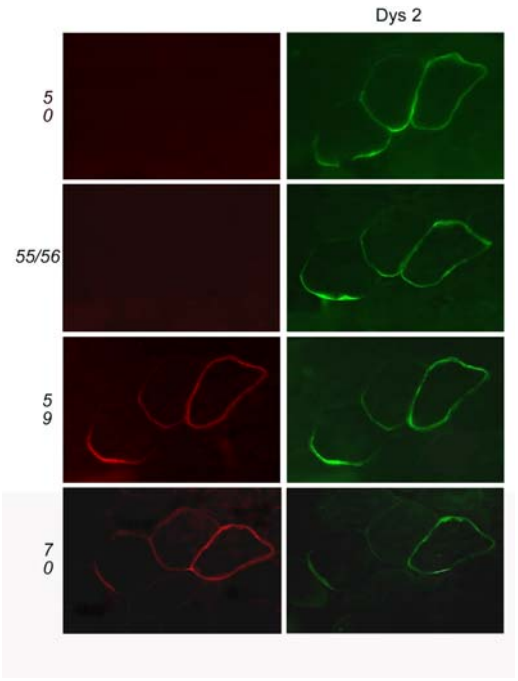
Visualization of mini-dystrophin and revertant dystrophin in muscle. Mini-dystrophin in vector-treated biceps muscle was assessed by immunofluorescence using an antibody (MANDYS-3)

that detects the N-terminus of dystrophin. Myocytes containing mini-dystrophin react with MANDYS-3 but not an antibody specific for the C-terminus of the protein, which is removed when dystrophin cDNA is miniaturized to accommodate the small insert capacity of AAV. Revertant dystrophin was identified by immunofluoresence with exon-specific antibodies. This approach facilitates mapping of dystrophin exons translated in the correct reading frame because of a spontaneous gene splicing event or a second site mutation that bypasses the genetic deletion.

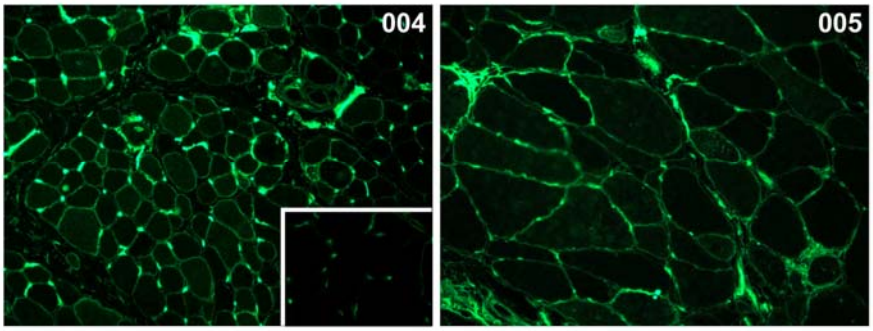
Supplemental Figures.



Supplementary fig. 1. Peripheral blood mononuclear cells (PBMC) collected from subject 004 at day 540 post vector treatment were incubated with mini-dystrophin peptide pool 3 (MDP3) or an irrelevant control peptide pool (CP). Depletion of CD4+ T cells from PBMC immediately before the IFN-γ ELISpot assay enhanced the response to MDP3, indicating that CD8+ T cells were the source of this cytokine.



Supplemental fig. 2. Exon profiling of biceps muscle from Subject 004. Muscle collected at day 42 after vector treatment was tested for reactivity with an antibody (MANDYS2) that recognizes the C-terminus of dystrophin (green, right panels). The left panels (red) show staining with antibodies specific for epitopes encoded by the indicated exon. Reactivity of antibodies directed against exon 59 and 70 encoded epitopes indicate expression of revertant dystrophin through the region targeted by MDP3-specific CD8+ T cells.



Supplemental fig. 3: Class I MHC Staining of Biceps Muscle. Biceps muscle sampled from patient 004 at day 42 (left panel) and patient 005 at day 90 (right panel) was stained with anti-class I antibodies conjugated to fluorescein isothiocyanate (FITC). The left panel inset shows reduced class I staining of the untreated biceps muscle from patient 004.